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13. ABSTRACT *(Maximum 200*

This grant consisted of 4 separate projects; Projects 1 and 4 aimed at assessing the association between lifestyle factors (exogenous hormone use) and breast cancer. The other two projects aimed at elucidating the role of estrogen metabolism in breast cancer development (Project 2) and the association between female hormones and mammographic densities, a possible intermediate endpoint for breast cancer (Project 3). Oral contraceptive use was not found to explain the increase in breast cancer risk that Asian migrants experience the first decade after they migrate to the US (Project 1). However, oral contraceptive use at a young age was associated with early onset breast cancer in young women (aged 35 or younger), while recent use appeared to be a more important risk factor in middle aged women (aged 36-40) (Project 4). Results from Project 2 suggest that the ratio of the two urinary estrogen metabolites 2-hydroxyestrone and 16α-hydroxyestrone does not differ between postmenopausal breast cases and controls or between premenopausal women at 'high' and 'normal' risk of breast cancer. Project 3 suggested that reducing serum-levels of hormones reduced the amount of mammographic densities. The results from the last project may prove useful for cancer prevention studies.

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(4) Table of Contents:

(5) Introduction:

Since projects 1 and 4 both aimed at elucidating the role of oral contraceptive in breast cancer development, project 4 has been discussed right after project 1.

Project 1: Case-control study of breast cancer in Asian-American

Breast cancer incidence has traditionally been 4-7 times higher in the US than in Asia (1-3). This discrepancy in rates has diminished over time, however, around 1985 breast cancer incidence rates were still 2.5-4 times higher in the U.S. than in the Philippines, Japan and China (1). There is substantial evidence that when Chinese, Japanese and Filipina women migrate to the U.S., their risk of breast cancer increases over several generations and approaches those of white U.S. women (4-8). Ziegler et al. reported that risk of breast cancer increased rapidly in a population of Asian immigrants the first decade after they arrived in the US (9). Such a rapid increase in risk may be due to changes in modifiable life style factors. However, although both reproductive (10), anthropometric (11) and dietary (12) factors were associated with risk of breast cancer in this population, changes in these factors could not explain all the increased risk of breast cancer occurring the first decade after migration to the US.

Oral contraceptive (OC) use represents another modifiable life-style factor. While early studies were largely reassuring, a number of more recent studies have found an association between long-term use of OCs and breast cancer in young women (13-23). A recent reanalysis of the majority of previously published studies found an increased risk associated with recent OC use (24, 25). Since OC use has been much lower in Asian countries than in the US (26, 27), we hypothesized that part of the increase in breast cancer risk that occurs among Asian women who migrate to the US could be due to OC use.

The research question for this project was to quantify the relative risk of breast cancer associated with oral contraceptive use in this population of Asian immigrants (9-12) and to examine how much of the difference in breast cancer risk observed in recent versus long-term immigrants could be explained by differences in patterns of oral contraceptive use.

Project 4: Hormone use and breast cancer in young women

The exact aspect of OC use that may increase the risk of breast cancer is not clear, although several studies have suggested that early use is important. The Collaborative Group reanalysis concluded that the most important determinant of breast cancer risk was recency of use (24, 25); however, the effect was strongest in the subgroup that started OC use at an early age. Since many of the studies that contributed data to this reanalysis were conducted a number of years ago, the collaborative study therefore did not focus on what particular aspects of OC use could be important for breast cancer development at an early age in the cohort of women who may have been exposed to OC use all of their reproductive life.

In this project I analyzed data from a population-based case-control study that was specifically designed to evaluate the relationship of breast cancer risk to particular patterns of OC use. In this study controls were matched to cases based on parity (nulliparous/parous), in order to provide optimal statistical power to evaluate effect modification by parity. Further, in order to

address the effects of OC use at an early age, the study was restricted to women 40 years of age or under at time of diagnosis (born in 1943 or later). These women were in their teens when OCs were introduced in the U.S. in the early 1960s.

Project 2a. Estrogen metabolism in breast cancer cases and controls

There is considerable evidence that increased serum estrogen levels are associated with an increased risk of breast cancer in postmenopausal women (28-38); and increased urinary excretion rates of estrogen (estrone, estradiol and estriol conjugates) in postmenopausal breast cancer cases compared to controls have also been consistently found (39-46). In addition to increased serum estrogen levels, it has also been suggested that the pathway by which estrone (E1) is metabolized may be important in determining breast cancer risk (47-48). Of the two main metabolic pathways for E1, the 16-hydroxy pathway yields biologically active products, while the 2-hydroxy pathway yields metabolites which are essentially devoid of estrogen activity (49-51). The suggestion is that an elevated rate of 16-hydroxylation as demonstrated by an increased ratio of urinary 16-alpha-hydroxyestrone (16αOHE1) to urinary 2-hydroxyestrone (2OHE1) is associated with an increased risk of breast cancer (47-48, 52-53). However, the epidemiologic data to support this hypothesis are sparse, consisting of a solitary case-control study of 9 perimenopausal and 24 postmenopausal breast cancer cases and 10 postmenopausal controls (52); where the ratio of 16αOHE1 to 2OHE1 was 31.3% in the breast cancer cases and 23.0% in the controls. If the ratio 16αOHE1/2OHE1 was a risk factor for breast cancer then this could be very important as regards future prevention strategies because there is evidence that the pathway of E1 metabolism may be altered by dietary (in particular, cruciferous vegetables) and other factors (54-58).

In this project we compared the ratio of urinary 16αOHE1 to 2OHE1 in postmenopausal breast cancer cases to that in postmenopausal controls.

Project 2b. Estrogen metabolism in women at high and low risk of breast cancer.

In a study by Osborne et al. (53), 17 women at 'high risk' of breast cancer (family history of breast cancer or epithelial atypia in previous biopsy) were compared with women without high risk lesions or a family history ('low-risk' controls). The comparison of urinary 16αOHE1 to 2OHE1 was very similar to that found in the case-control study of Schneider et al. (52). No further details regarding the study subjects were provided, and no other studies have been reported attempting to confirm or refute this finding.

In this project we compared the ratio of urinary 16αOHE1 to 2OHE1 in women with a strong family history of breast cancer to that in women with no such history.

As 'cases' we used premenopausal daughters or sisters of women diagnosed with either premenopausal bilateral breast cancer before the age of 50, or unilateral breast cancer before the age of 40.

Project 3: Changes in mammographic densities in women on a gonadotropin-releasing hormone agonist contraceptive regimen.

Although certain serum levels of ovarian steroid hormones are necessary for optimal health, premenopausal women who are not trying to conceive appear to require considerably less of these hormones than is produced by the ovary (59-60). Spicer and Pike have developed a gonadotropin releasing hormone (GnRH) agonist plus ultra-low-dose estrogen and ultra low-dose progestogen hormonal contraceptive regimen, that attempts to reduce the levels of estrogen and ultra-low-dose progestogen to a minimum, while still preserving the essential beneficial effects of estrogen (60-61). With the use of ultra-low-dose estrogen and progestogen alone ovarian function is not blocked by these sex steroids as with standard hormonal contraceptives; blocking of ovarian function is achieved through the use of the GnRH agonist (GnRHA) which results in the suppression of pituitary follicle stimulating hormone (FSH) and luteinizing hormone (LH) release. Sufficient estrogen is given to prevent hypo-estrogenic symptoms (such as hot flashes); and intermittent progestogen is given to prevent any estrogen-induced endometrial hyperplasia. Since the GnRH agonist also blocks testosterone production from the ovary, sufficient testosterone is also included in the regimen to just replace that no longer produced by the ovary.

The GnRH based regimen substantially lowers the levels of female sex hormones. Such a regimen may provide long-term reduction in breast cancer risk by reducing breast cell division (59-61). Mammographic densities have been demonstrated to be significantly associated with breast cancer risk, independent of other breast cancer risk factors, with higher densities being associated with up to a 5-fold increase in risk (62-65). Mammographic densities appear to decline with menopause (64) and increase in women who take hormonal replacement therapy.

We previously described reductions in mammographic densities in women on this regimen (66). However, in this study we used a subjective comparative method to determine changes in mammographic densities. The question remained whether there would be observable changes in densities if a quantitative method was used. We wanted to determine if a new computer-assisted method as well as a more traditional outlining method could detect these fine changes in mammographic densities.

(6) Body

Project 1: Case-control study of breast cancer in Asian-Americans

METHODS

This project was based on data from a completed case-control study where cases were all women of Chinese, Japanese or Filipina ethnicity diagnosed with histologically confirmed breast cancer between age 20 and 55 in San Francisco, Los Angeles and Hawaii between 1983 and 1987 (9-12). Controls were selected by random digit dialing methods in Los Angeles and San Francisco, and from the annual household sample survey in Hawaii. The data set contains information on 597 cases and 966 controls. An in person interview was conducted, using standardized questionnaires with questions on residence history, menstrual, reproductive history, anthropometric variables and diet at three different time periods. Details can be found in Ziegler et al., (9). Odds ratios of breast cancer associated with oral contraceptive use were estimated using logistic regression, adjusting for potential confounders in the model (67). Statistical analyses were performed using SAS (SAS Institute Inc., Cary, NC) and EPILOG (Epicenter Software, Pasadena, CA).

RESULTS

Oral contraceptive (OC) use increased with time since migration; 14.7% of Asian born women who had been here less than 8 years, 33.4% of Asian born women who had been here 8 years or longer, and 49.6% of women born in the US had ever used OCs. However, duration of OC use (adjusted for age, ethnicity, location and family history of breast cancer) was not associated with increased risk of breast cancer. Moreover, OC use before age 25 or first pregnancy was not associated with increased risk. Results were unchanged when restricted to women under age 45.

We also examined whether there could be effect modification by certain demographic variables such as ethnicity, residence (study location), years since migration and age at diagnosis. There was no apparent effect modification by ethnicity, study location or age at diagnosis. However, for years since migration we observed the highest OR associated with long term OC use among recent migrants. These findings were however, based on small numbers (10 OC using cases, and 21 OC using controls), and were not statistically significant.

Migrants who had been here 8 years or more were still at twice the risk of women who had been here less than 8 years when duration of OC use was incorporated into the model. This study suggests that increased oral contraceptive use cannot explain the rapid increase in breast cancer risk that occurs among Asian women who migrate to the US (9).

The manuscript for this project will be submitted shortly.

Project 4: Hormone use and breast cancer in young women

METHODS

The methods of this study have been described in detail (69, 70). Briefly, the cases were white female residents of Los Angeles County, aged 40 years or younger, diagnosed with in situ

or invasive breast cancer between July 1, 1983 and January 1, 1989. The Los Angeles County Cancer Surveillance Program, the population-based cancer registry for the county, identified 969 eligible breast cancer patients, and interviews were completed with 744. One neighborhood control was individually matched to each of the 744 interviewed cases on birth date (within 36 months), race (white), parity (nulliparous versus parous) and neighborhood of residence. Eligibility (for both case patients and neighborhood controls) was restricted to women born in United States, Canada or Europe.

In-person interviews were conducted with all subjects by the same female nurse-interviewer. We obtained complete reproductive, contraceptive and physical exercise histories on all subjects up to the date of the case patient's diagnosis. This date has been used as the cutoff date for information used in the analyses represented here.

The data were analyzed using univariate and multivariate conditional logistic regression methods for individually matched case-control studies (67).

RESULTS

Compared to no use, having used OCs for 12 years or more was associated with a modest non-significant elevated breast cancer risk with an odds ratio (OR) of 1.4 (95% confidence interval (CI) = 0.8-2.4). However, long-term (12 years or more) users of high-dose estrogen pills had 60% higher breast cancer risk than never users (CI = 0.9-3.2). Further, women aged 35 years or younger who had used OCs for a year or more before age 18 had nearly a two-fold elevated breast cancer risk (OR = 1.9, CI = 0.9-3.9). The effect of recent use appeared to be limited to women diagnosed after age 35. Analyses by parity and stage yielded similar results (71). The manuscript has been submitted.

Project 2a. Estrogen metabolism in breast cancer cases and controls

METHODS

I was awarded a small grant to perform this study (DAMD17-94-J-4289). Postmenopausal subjects, who participated in an ongoing case-control study of breast cancer at our institution (NIH grant: 5 P01 CA17054) were eligible for inclusion in this study. Exclusion criteria were: having ever been treated with chemotherapy, or been diagnosed with systemic lupus erythematosus or liver cirrhosis; having smoked the previous 3 years; having the past 6 months used medications that may interfere with estrogen metabolism (estrogen, progesterone, tamoxifen, cimetidine, carbamazepin, phenytoin, barbiturates, thyroxin, corticosteroids or omega-3 fatty acid supplements); having the past 3 months had general anesthesia; and currently weighing more than 200 lbs. The cases in the case-control study were incident cases of histologically confirmed breast cancer, and were ascertained through the population based cancer registry for Los Angeles County (Los Angeles County/University of Southern California Cancer Surveillance Program, LACCSP, a NCI SEER registry). Controls were matched to cases by age and neighborhood of residence. (Matching was ignored in the current study).

We contacted a total of 475 cases and 445 controls who were listed as non-smokers and who had a weight of 200 lbs or less on the original questionnaire. Of the women contacted, only 20% were eligible, 25% were not found and the rest were ineligible. Main reasons for ineligibility included current tamoxifen use among the cases

(approximately 30%) of all cases contacted, current estrogen use among controls (38% of all controls contacted, and other medications (10-13%). Refusals were 5%.

Early morning urine samples were collected from 75 breast cancer cases and 89 controls. The following urinary metabolites were determined: 16αOHE1, 2OHE1, estrone (E1), estradiol (E2) and estriol (E3). The 16αOHE1 and 2OHE1 were determined by enzyme immunoassay by Dr. Leon Bradlow at the Strang-Cornell Cancer Research Laboratory in New York. E1, E2 and E3 conjugates were determined by radioimmunoassay in the laboratory of Dr. Frank Stanczyk at Los Angeles County/USC Women's Hospital. Data on current body weight, recent diet and alcohol consumption were also collected in order to adjust for these potential confounders in the analyses.

Results were analyzed statistically using t-tests and standard analyses of covariance techniques, as well as logistic regression (67, 72). The values of the hormone measurements were log transformed before analyses to achieve approximate normality of results. In the logistic regression, the odds ratio per unit increase in 16αOHE1, 2OHE1 and 16αOHE1/2OHE1 were calculated, with adjustment of potential confounders.

RESULTS

The mean 16α-OHE1 was 17% higher and 2-OHE1 was 19% higher in cases than in controls. The ratio of 16α-/2-OHE1 was 8% higher in cases than in controls; none of these findings were statistically significant. Mean levels of E1, E2, E3 were 21%, 13% and 9% higher in cases than in controls, none of these findings were statistically significant. Preliminary results from this study were published earlier this year (73, 74). A more extensive report is in preparation.

Project 2b. Estrogen metabolism in women at high and low risk of breast cancer.

METHODS

I was awarded a small grant to perform this study (DAMD17-94-J-4231). 'Cases' in this study were premenopausal sisters and daughters of patients with a) premenopausal bilateral breast cancer who participated in a genetic-epidemiologic study (75), or b) unilateral breast cancer before age 40 who participated in a breast cancer case-control study (69, 70). Eligible women had never themselves been diagnosed with breast cancer. 'Controls' were daughters or sisters of women participating as controls in the breast cancer case-control study (69, 70) or in the Los Angeles part of the national Women's Contraceptive and Reproductive Experiences (CARE) study (P.I. Leslie Bernstein). The exclusion criteria were the same as in project 2a. In addition we excluded women who were pregnant or breast feeding, or who had irregular menstrual cycles.

We contacted 543 women with premenopausal uni- or bilateral breast cancer and 744 controls who participated in one of our previous studies (see above). Initial attempts were made to contact these women by telephone. Subsequent contacts were made by mail and/or telephone. We sent up to four letters to some of these women. Women who's current address was unknown (their letters have been returned with no forwarding address), were attempted tracked through the records of the California Department of Motor Vehicles, HANES inverse street directory, the Voter's Registry, the California Mortality Registry and TRW. An 800-number was set up to

increase response rates. We also sent an interviewer to the last known address of a random sample of 20 non-responders in order to find out their reason for not responding. All women reached indicated that they had no sister or daughter and therefore saw no reason for returning our phone calls/letters.

A total of 128 cases and 125 controls had at least one daughter or sister between the ages of 20 and 50 living in California (total of 254 case daughters or sisters and 208 control daughters or sisters). We contacted all of these case and control daughters and sisters. When there was more than one daughter/sister in each family, we include the youngest one above age 20 if eligible. We obtained responses from 183 case daughters or sisters (72%) and 140 control daughters or sisters (67%). A total of 83 case daughters or sisters and 29 control daughters or sisters have been found to be eligible. Major reasons for ineligibility include current oral contraceptive use (40%), current smokers (15%), other medications (10%), irregular periods (10% of controls), currently pregnant/breast feeding (10% of controls). We collected urine samples and dietary questionnaires on 76 case daughters or sisters and 27 control daughters or sisters.

RESULTS

There were no statistically significant differences in mean 16α-OHE1, mean 2-OHE1 or the ratio 2-OHE1/16α-OHE1 between cases than in controls. The mean ratio of 2-OHE1/16α-OHE1 was 1.84 among controls and 1.78 among cases ($p = 0.80$). There were no statistically significant differences in mean levels of E1, E2 and E3. A manuscript describing the major findings from this study should be submitted before the end of the year.

Project 3: Changes in mammographic densities in women on a gonadotropin-releasing hormone agonist contraceptive regimen

METHODS

The extent of mammographic densities was determined by 1) a reader with substantial experience in outlining mammographic densities using Wolfe's outlining method (76, 77), and 2) our new computer-assisted method of determining densities. The results of both these methods were compared with our previously described method (66).

For the computer-assisted method, all mammograms were digitized using an Omnimedia XRS 6cx scanner (Lumisys, Sunnyvale, CA).

Our computer-assisted method of determining densities was based on the method developed by Byng and colleagues (78), and the software was developed by a physicist (Dr. Mel Astrahan) at USC. This new software program works as follows: On the digitized mammographic image displayed on the screen, the reader first defines a region of interest (ROI) for analysis; this region includes all of the breast shown on the mammogram, but specifically excludes the pectoralis muscle (same area used for Wolfe's method). The reader subsequently uses a tinting tool to color yellow all pixels she (or he) considers represent mammographic densities. We use a system based on 256 different gray levels, with 0 being the darkest value and 255 the whitest. The user colors yellow the pixels between 255 and X (where X is any color value selected by the user between 0 and 255) using the tinting tool. The pixel count

(corresponding to the area colored) can then be recorded from the screen. The percentage of the breast containing densities can then be estimated.

The mammograms were read 3 times with the computer-assisted method. For the comparison between the methods described here, the results from the first of these 3 readings were used.

Statistical analysis of this study were done using standard methods (67,72). Statistical analyses will be performed using the SAS (SAS Institute Inc., Cary, NC) and EPILOG (Epicenter Software, Pasadena, CA).

RESULTS

Both Wolfe's outlining method and the computer-assisted method showed high consistency with the previous method we have used to classify mammographic densities. All three methods yielded statistical significant reductions in densities from baseline to the 12-month follow-up mammogram in women on the contraceptive regimen. The differences between the control and the treated group were statistical significant with Wolfe's outlining method, and was of borderline statistical significance with the computer-assisted method. The computer-based results correlated highly ($r>0.85$) with results from Wolfe's outlining method. These results suggest that our simple computer-based method of determining mammographic densities is highly reproducible and correlates well with Wolfe's outlining method of mammographic densities. Both the computer-based method and Wolfe's outlining method can detect fine changes in mammographic densities. The manuscript describing these findings is in press (79).

(7) Conclusion (of all five projects)

Results from this project are compatible with a role of hormones in breast cancer etiology. Oral contraceptive use could not explain the rapid increase in breast cancer risk in recent immigrants from Asia. However, interestingly, the study suggests that the effect of OC use may be highest in recent migrants. Results from the case-control study of early onset breast cancer are consistent with a modest effect of early OC use on breast cancer risk in the very young women. The reason why women in the youngest age group appear to be at particularly high risk if they have used OCs at an early age must be further explored in future studies.

Results from the two projects on estrogen metabolism suggest that the ratio of the two urinary metabolites, 2-OHE1 to 16α-OHE1 does not represent a good biomarker for breast cancer risk that would be useful in breast cancer prevention. On the other hand, the results from the mammographic density study adds to the growing evidence that mammographic densities may indeed be a useful surrogate endpoint for breast cancer risk. Others have found mammographic densities to be strongly associated with breast cancer risk, and also with exogenous hormone use. Although further research is needed, mammographic densities may prove to represent a surrogate endpoint for breast cancer that could be useful in cancer prevention studies.

As a result of this completed project, I am aiming my future breast cancer research in two directions: 1) to further understand the role of exogenous hormones, and 2) to gather additional data to determine if mammographic density can be used as a surrogate endpoint for breast cancer risk that could be useful in cancer prevention studies.

Specifically, I am working towards further understanding why some young women are at increased risk of breast cancer if they have used oral contraceptives, in particular to determine whether the presence of a BRCA1 mutation will alter the effect of OCs on breast cancer risk. I plan to answer this question by conducting a large case-case study in Los Angeles (an NIH proposal is pending).

We have recently been funded to conduct a study to determine whether mammographic densities are predictive of breast cancer in twins, to what extent mammographic densities are heritable, and what role environmental factors may play. We also have been funded to assess whether mammographic density represents a risk factor in three different ethnic groups (African Americans, Asian Americans and Whites), and to assess the association between other breast cancer risk factor and mammographic densities.

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(9) Appendix

a) **Personnel receiving pay from this effort:**
Giske Ursin, MD, Ph.D.

b) **Bibliography of publications and meeting abstracts:**

PUBLICATIONS (1 and 2 enclosed):

1. Ursin G, London S, Stanczyk FZ, Gentzschein E, Paganini-Hill A, Ross RK, Pike MC. A pilot study of urinary estrogen metabolites (16α-OHE1 and 2-OHE1) in postmenopausal women with and without breast cancer. Environmental Health Perspectives 1997; 105(suppl) 601-605.

2. Ursin G, Astrahan MA, Salane M, Parisky YR, Pearce JG, Daniels J, Pike MC, Spicer DV. The detection of changes in mammographic densities. Cancer Epidemiology Biomarkers and Prevention (in press).

3. Ursin G, Bernstein L, Sullivan-Halley JA, Hanisch R, Ross RK. Oral contraceptive use and breast cancer risk in young women (submitted).

4. Ursin G, Ziegler RG, Pike MC, Wu AH, Hoover RN, West DW, Nomura AMY. Breast cancer in Asian American women - no relation with oral contraceptive use (will be submitted shortly).

MEETING ABSTRACTS:

1. Ursin G, Bernstein L, Sullivan-Halley JA, Hanisch R, Ross RK. Oral contraceptive use and breast cancer risk in women aged 40 or younger. Am J Epidemiol 1996; 143:43s.

2. Ursin G, Ziegler RG, Pike MC, Wu AH, Hoover RN, West DW, Nomura AMY. Oral contraceptive use and breast cancer risk among Asian-American women. Am J Epidemiol 1995;141:52s.

3. Ursin G, London S, Stanczyk FZ, Gentzschein E, Paganini-Hill A, Ross RK, Pike MC. Are estrogen metabolites biomarkers for breast cancer risk? Proceedings of the American Assoc Cancer Research 1997;38: 619.

A Pilot Study of Urinary Estrogen Metabolites (16α-OHE_1 and 2-OHE_1) in Postmenopausal Women with and without Breast Cancer

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The two main pathways for metabolizing estrogen are via 16α-hydroxylation and 2-hydroxylation. The 16α-hydroxy metabolites are biologically active; the 2-hydroxy metabolites are not. It is suggested that women who metabolize a larger proportion of their endogenous estrogen via the 16α-hydroxy pathway may be at significantly elevated risk of breast cancer compared with women who metabolize proportionally more estrogen via the 2-hydroxy pathway. In particular, it is suggested that the ratio of urinary 2-hydroxyestrone (2-OHE_1) to 16α-hydroxyestrone (16α-OHE_1) is an index of reduced breast cancer risk. This pilot study compared this ratio in postmenopausal women diagnosed with breast cancer to those of healthy controls. Urinary concentrations of estrone (E_1), 17β-estradiol (E_2) and estriol (E_3) were also quantified. White women who were subjects in a previous breast cancer case–control study at our institution were eligible for inclusion. All participants provided a sample of their first morning urine. The results from the first 25 cases and 23 controls are presented here. The ratio of 2-OHE_1 to 16α-OHE_1 was 12% lower in the cases ($p=0.58$). However, urinary E_1 was 30% higher ($p=0.10$), E_2 was 58% higher ($p=0.07$), E_3 was 15% higher ($p=0.48$), and the sum of E_1, E_2, and E_3 was 22% higher ($p=0.16$) in the cases. These preliminary results do not support the hypothesis that the ratio of the two hydroxylation metabolites (2-OHE_1/16α-OHE_1) is an important risk factor for breast cancer or that it is a better predictor of breast cancer risk than levels of E_1, E_2 and E_3 measured in urine. — *Environ Health Perspect* 105(Suppl 3):601–605 (1997)

Key words: estrogen metabolism, 16α-hydroxyestrone, 2-hydroxyestrone, breast cancer, urinary estrogen metabolites

Introduction

Overwhelming evidence supports a role of ovarian hormones in the etiology of breast cancer (*1*). At menopause circulating estrogens decline sharply, explaining in large part, and possibly completely, the decreased breast cancer risk associated with early menopause (*2*). In postmenopausal women, the major source of estrogen arises from the peripheral conversion of androstenedione in adipose tissue (*3*). This, together with decreased sex hormone-binding globulin levels, is the most probable explanation for the higher breast cancer risk in obese postmenopausal women (*4*). Both elevated serum estrogen levels (*5–16*) and increased urinary excretion rates of estrone (E_1), 17β-estradiol (E_2) and estriol (E_3) have been found in breast cancer cases as compared with controls (*17–24*).

The two main pathways for metabolizing estrogen are via 16α-hydroxylation and 2-hydroxylation, and the major estrogen metabolites excreted in urine are 2-hydroxy products [2-hydroxyestrone (2-OHE_1), 2-hydroxyestradiol (2-OH-E_2), 2-methoxyestrone (2-MeO-E_1)], nonmetabolized E_1, 16α-hydroxy products [E_3, 16α-hydroxyestrone (16α-OHE_1)]and E2 (*25*). The 16α-metabolites are biologically active (*26,27*); the 2-hydroxy metabolites are not (*28*).

The extent to which estrogen is metabolized via the 16α-hydroxylation pathway may be associated with breast cancer risk (*29–31*). Increased 16α-hydroxylation activity, but not 2-hydroxylation activity, has been observed in mice strains with high spontaneous mammary tumor formation (*29*). In humans, the extent of biotransformation of ^{3}H-E_2 via the 16α-hydroxylation pathway was 4.6-fold higher in terminal duct lobular units in breast tissue from breast cancer cases than in breast tissue from reduction mammoplasty controls (*32*). Two other epidemiologic studies suggested that the extent of 16α-hydroxylation was higher in women with breast cancer (*33*) and in women with high familial risk of breast cancer (*34*) than in controls. However, a third study found no elevation of 16α-hydroxylation in breast cancer cases compared with controls (*25*).

We selected women interviewed in a previous population-based epidemiologic study to determine whether postmenopausal women with breast cancer have a lower ratio of urinary 2-OHE_1 to 16α-OHE_1 than controls. We report here the data from the first 25 cases and 23 controls.

Methods

This study was approved by the local Institutional Review Board. Written informed consent was obtained from each participant.

Eligible cases were identified from women between 55 and 64 years of age

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Abbreviations used: E_1, estrone; E_2, 17β-estradiol; E_3, estriol; EIA, enzyme immunoassay; HPLC, high-performance liquid chromatography; 2-OHE_1, 2-hydroxyestrone; 16α-OHE_1, 16α-hydroxyestrone; RIA, radioimmunoassay.

diagnosed with histologically confirmed breast cancer, identified through the Los Angeles County Cancer Surveillance Program (a National Cancer Institute Surveillance, Epidemiology, and End Results Program Registry), who had participated in a previous breast cancer case–control study conducted at our institution (*35*). The dates of diagnosis were 1 March 1987 through 31 December 1989. Only women diagnosed with incident cancer at stage II or less [tumor size $\leq T_2$, nodes $\leq N_1$, and no distant metastasis (M_0), or T_3, N_0, M_0] were included (*36*). Eligible controls were participants in the same case–control study who had not been diagnosed with breast cancer. Subjects had to be English-speaking whites (including Hispanics), and residents of Los Angeles County.

Cases and controls were contacted; the most recent interviewees were contacted first. Eligibility was determined based on a phone interview. Subjects who had used medications during the previous 6 months that may have interfered with estrogen metabolism (specifically, cimetidine, thyroxine, estrogen, progesterone, tamoxifen, or ω3 fatty acid supplements) (*37–40*) were eliminated from the study. Subjects who had general anesthesia in the previous 3 months or weighed more than 200 lb (90 kg) were also excluded.

A box containing a 100-ml urine vial with a 100-mg ascorbate tablet, a small cooler with an ice pack, an informed consent form, and a questionnaire on recent intake of medication, alcohol, and specific foods was shipped to each eligible woman who agreed to participate. First morning urine samples were collected, aliquoted, and frozen at –70°C within 6 hr after specimens were produced.

Urine samples were sent to two different laboratories. Batches of 30 samples (15 from cases, 15 from controls, including 10% duplicates) were coded and shipped on dry ice. The only identifiers on the samples were code numbers ensuring that the laboratories were blinded as to case or control status of the individual samples and to the identity of duplicates.

Enzyme Immunoassay of 16α-OHE_1 and 2-OHE_1

Measurements of urinary 16α-OHE_1 and 2-OHE_1 were carried out using commercially available competitive enzyme immunoassay (EIA) kits (Estramet, Immuna Care Corporation, Bethlehem, PA) to measure 2-OHE_1 and 16α-OHE_1 directly in urine. The two metabolites were measured simultaneously to avoid interassay variation. This method has been described in detail by Klug et al. (*41*). In brief, monoclonal antibodies to the estrogen metabolites were immobilized directly to the solid phase, and the metabolite standards were conjugated to alkaline phosphatase enzyme. Each urine sample was acidified and subjected to β-glucuronidase/aryl sulfatase hydrolysis before assay.

The 16α-OHE_1 and 2-OHE_1 EIA kits were validated by comparing values obtained with these kits to values obtained by gas chromatography–mass spectroscopy (*41*). The inter- and intraassay coefficients of variation for 2-OHE_1 and 16α-OHE_1 were between 7 and 13% (*41*). Creatinine values above 0.20 mg/ml are considered necessary to obtain adequate reproducibility of the 2-OHE_1 and 16α-OHE_1 assays (HL Bradlow, personal communication).

Radioimmunoassay of Urinary E_1, E_2, and E_3

Measurements of urinary E_1, E_2, and E_3 were carried out using high-performance liquid chromatography–radioimmunoassay (HPLC–RIA). Each urine sample was acidified and subjected to β-glucuronidase/aryl sulfatase hydrolysis before assay.

Following the addition of approximately 1000 dpm of ^{3}H-E_1, ^{3}H-E_2, and ^{3}H-E_3, which served as internal standards to follow procedural losses, solid phase extraction was performed. Ethyl acetate was used to extract the estrogens, the organic solvent was evaporated and the extract was subjected to HPLC. A reverse-phase HPLC column (C_{18}; 5μ) was used to elute E_3, E_2, and E_1 in a gradient of acetonitrile:water:acetic acid (40:60:0.1) at a flow rate of 1 ml/min. The retention times for E_3, E_2, and E_1 were 4, 13, and 16 min, respectively.

The E_1, E_2, and E_3 fractions were quantified by RIA, using methods previously described by Katagiri et al. (*42*), Stanczyk et al. (*43*), and Cassidenti et al. (*44*). Appropriate quality controls were used with each set of samples that was assayed to monitor assay reliability.

Statistical Analysis

All directly measured hormone variables were lognormally distributed, and the statistical significance of the difference in these variables between cases and controls was evaluated using *t* tests of the natural logs of these values. The statistical significance of the differences in 2-OHE_1/16α-OHE_1 between cases and controls was evaluated using Wilcoxon's nonparametric rank sum test. Statistical analyses were conducted using SAS (SAS Institute, Cary, NC).

Results

The full study will include almost 100 cases and 100 controls. We reported here results from the first subset of the women enrolled in the study.

The results for the first two batches of urine samples were available for the analyses reported here. These represented 27 cases, 27 controls, and 6 duplicate samples. We excluded six samples with low creatinine values. Among the remaining 25 cases and 23 controls, the mean 16α-OHE_1 was 8.0% higher and the mean 2-OHE_1 was 3.9% lower in cases than in controls (Table 1). The ratio of 2-OHE_1 to 16α-OHE_1 was 12.0% lower in cases. None of these differences were statistically significant. The individual values of 2-OHE_1/16α-OHE_1 are plotted in Figure 1.

Ratios of 2-OHE_1/16α-OHE_1 below 2.0 have been suggested as an index of high risk of breast cancer (HL Bradlow, personal communication). However, in this study, nearly all cases and controls had at least this low ratio; 20 of 23 controls and 24 of 25 cases had ratios less than 2.0.

E_1 was 30% higher ($p = 0.10$) and E_2 was 58% higher ($p = 0.07$) in cases than in controls. E_3 was 15% higher and the sum of E_1, E_2, and E_3 was 22% higher in cases; neither result was statistically significant.

Table 1. Mean levels of estrogen metabolites in postmenopausal breast cancer cases and controls.

Urinary metabolite[a]	Cases, *n* = 25		Controls, *n* = 23		Difference,[b] %	*p* value
	Mean	SE	Mean	SE		
2-OHE_1	7.09	0.89	7.38	0.77	–3.9	0.89
16α-OHE_1	5.27	0.47	4.88	0.37	8.0	0.61
2-OHE_1/16α-OHE_1	1.39	0.10	1.58	0.20	–12	0.58
E_1	3.14	0.34	2.42	0.34	30	0.10
E_2	0.87	0.14	0.55	0.06	58	0.07
E_3	5.63	0.66	4.90	0.49	15	0.48
$E_1+E_2+E_3$	9.64	0.94	7.87	0.81	22	0.16

[a]ng/(mg creatinine). [b][1–(cases mean value)/(controls mean value)] × 100.

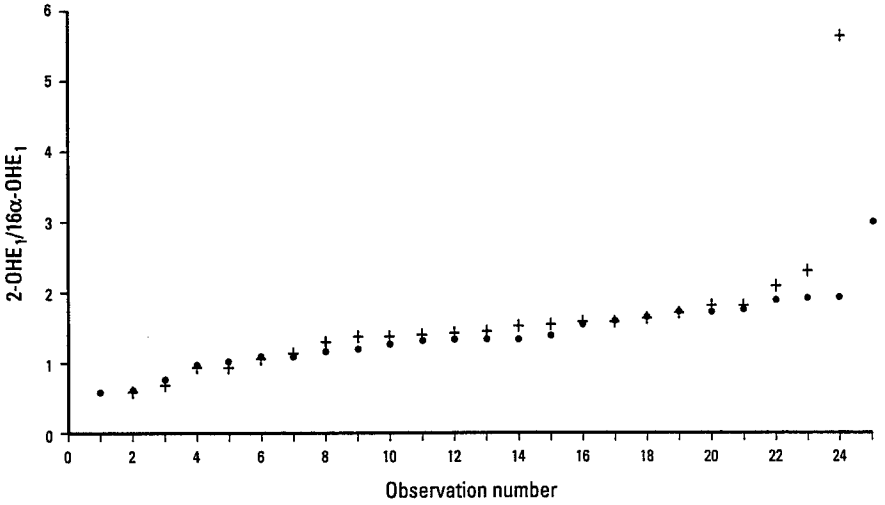

Figure 1. Urinary 2-OHE_1/16α-OHE_1 in 25 postmenopausal breast cancer cases (•) and 23 controls (+).

The coefficients of variation for the six blind duplicates were 13% for 2-OHE_1, 20% for 16α-OHE_1, 13% for E_1, 14% for E_2, and 24% for E_3.

Discussion

Our results confirm previous studies that E_1 and E_2 are higher in urine of postmenopausal breast cancer cases than controls (*17–24*). However, we found only small differences between cases and controls in urinary levels of 16α-OHE_1, 2-OHE_1, and the ratio of the two.

The epidemiologic data addressing the 2-OHE_1/16α-OHE_1 hypothesis are sparse. Schneider and co-workers used a radiometric method to determine the extent of 2- and 16α-hydroxylation (*33*). They injected 33 peri- and postmenopausal breast cancer patients and 10 postmenopausal controls with E_2 tracers labeled with ^3H in the 17α, C-2, and 16α position. They drew serial blood samples before and after isotope administration and determined the rate and extent of the oxidative metabolism at positions 17α, C-2, and 16α. Cases had 60% higher extent of 16α-hydroxylation than controls; this difference was statistically significant. However, the two groups did not differ significantly in the extent of 2-hydroxylation, which was only 5% higher among cases. The ratio of the average level of 16α-hydroxylation to the average level of 2-hydroxylation was 52% greater in the breast cancer cases than in the controls. No data on total estrogen values were provided.

The only other published study of 16α-/2-hydroxylation in breast cancer patients was performed by Adlercreutz et al. (*25*). They examined estrogen metabolites in young Finnish premenopausal breast cancer cases ($n = 10$) and control women on an omnivorous normal Finnish diet ($n = 12$) or on a lacto-vegetarian diet ($n = 11$). There was no statistically significant difference in 2-OHE_1, 16α-OHE_1, or total urinary estrogens (E_1, E_2, E_3, 2-OHE_1, 16α-OHE_1, and eight other estrogen metabolites) between breast cancer patients and omnivores or breast cancer patients and lacto-vegetarians.

Both of the above-mentioned studies measured metabolites after breast cancer diagnosis. In an attempt to determine whether an elevated ratio of 16α- to 2-hydroxylation precedes diagnosis, Osborne and co-workers used radiometric methods to study estrogen metabolism in premenopausal women presumed to be at high or low risk of breast cancer (*34*). They found that women at high risk of breast cancer (family history of breast cancer or epithelial atypia in a previous biopsy) had a significantly higher (22%) extent of 16α-hydroxylation than women without high-risk lesions or a family history (low-risk controls). High-risk women had a similarly elevated extent of 16α-hydroxylation of E_2 as the breast cancer patients in the study by Schneider et al. (*33*). Translated to relative risks, the data of Osborne et al. (*34*) suggest that one standard deviation increase in the extent of 16α-hydroxylation from the level of low-risk controls may result in a 3-fold elevation of breast cancer risk. No data on total estrogen values were provided.

Several factors could also have affected our results. We studied a select group of women with few extraneous factors that might influence estrogen metabolism. With this approach we excluded a large number of women. Based on the first 300 women identified, we excluded 55 to 60% for a variety of reasons: 10% were above 200 lb, 15% were smokers, 25% of controls were taking estrogen replacement therapy, 10% were on other medications, and at least 20% of the cases were on tamoxifen. However, none of these exclusions appear likely to introduce any biases in any direction because they were applied equally to cases and controls.

The intraassay coefficients of variation for the assays used in this study were 13 and 20%, respectively. These values are somewhat higher than the published values of approximately 10% (*41*). It is, however, unclear whether the original reproducibility tests were conducted in pre- or postmenopausal women. Ziegler (*45*) addresses reproducibility problems elsewhere in this volume. She found that the reproducibility of this assay was low when testing urines with low estrogen concentrations. As a result of these findings, both the 2-OHE_1 and 16α-OHE_1 tests are being adjusted to improve reproducibility at low concentrations (HL Bradlow, personal communication).

The evidence is rather clear that certain diets influence the extent of 16α- and 2-hydroxylation (*46–49*). Recent dietary changes in cases–controls could obscure or accentuate the differences between these groups. We addressed this issue by asking participants whether they have changed their diet in the past 10 years, and we will include a complete analysis of these data in a subsequent report on the completed study.

It is not known whether the onset of cancer may affect 2- and 16α-hydroxylation. We are therefore conducting another study examining the association between the extent of 2- and 16α-hydroxylation and familial risk of breast cancer in healthy young women.

In conclusion, our preliminary results from this case–control study of breast cancer in postmenopausal women do not support the hypothesis that the ratio of urinary 2-OHE_1 to 16α-OHE_1 is a better predictor of breast cancer risk than urinary E_1, E_2, and E_3.

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The Detection of Changes in Mammographic Densities

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Running title: Measuring mammographic density changes

ABSTRACT

We previously reported reductions in mammographic densities in women participating in a trial of a gonadotropin-releasing hormone agonist (GnRHA) based regimen for breast cancer prevention. In our previous report we compared (by simultaneous evaluation) 3 basic 'elements' of mammographic densities. The purpose of the present study was to evaluate whether a 'standard' (expert) method of measuring mammographic densities would detect such changes in densities, and whether a novel non-expert computer-based threshold method could do so.

Mammograms were obtained from 19 women at baseline and 12 months after randomization to the GnRHA-based regimen. The extent of mammographic densities was determined by (1) a standard expert outlining method developed by Wolfe and his colleagues, and (2) a new computer-based threshold method of determining densities.

The results from both the expert outlining method and the computer-based threshold method were highly consistent with the results of our original (simultaneous evaluation) method. All 3 methods yielded statistically significant reductions in densities from baseline to the 12-month follow-up mammogram in women on the contraceptive regimen. The difference between the treated and the control group was statistically significant with the expert outlining method, and was of borderline statistical significance with the computer-based threshold method. The computer-based results correlated highly ($r>0.85$) with the results from the expert outlining method.

Both the standard expert outlining method and the computer-based threshold method detected the reductions we had previously noted in mammographic densities induced by the GnRHA-based regimen.

INTRODUCTION

Wolfe (1,2) originally described 4 'parenchymal patterns' (N1, P1, P2, DY) of increasing mammographic densities and found that the risk of incident breast cancer increased with increasing density pattern and was much greater in women with the DY pattern than in women with the N1 pattern (2). Other investigators reported similar success with systems where the reader simply estimated from the mammogram the proportion of the breast containing glandular or ductal densities (3-5).

Wolfe et al. (6) subsequently described a method of actually measuring the extent of mammographic densities. In this method experienced readers (experts) outline the area with densities directly on the mammogram. The area containing densities and the total area of the breast are subsequently measured with a planimeter, and the % of the breast containing densities calculated. The % of the breast containing densities determined by this method has been found to be highly associated with risk of breast cancer (6-8).

Byng et al. (9) have developed a computer-based threshold method for measuring densities. Mammograms are digitized using a high-resolution laser scanner, and the scanned image is displayed on a computer screen. The reader selects a threshold value (gray scale on the computer screen) that best distinguishes the breast from the dark background. An edge detection computer program (algorithm) is used to outline the breast, and the computer measures the total area of the breast (in terms of computer screen pixels). Subsequently the reader identifies a second threshold value, the gray value that best identifies the edges of the mammographically dense areas within the breast parenchyma. The number of pixels in the areas containing such densities is then measured by the computer, and the fraction of pixels in the breast parenchyma that are located in the dense areas (% mammographic densities) is calculated. This computer-based threshold method has been found to be equally reproducible for experienced and inexperienced readers (9), and to be strongly predictive of breast cancer risk (10).

Our goal was to develop a method of determining mammographic densities that could be used in epidemiologic studies by personnel with minimal or no radiology training, and that could be implemented on relatively reasonable equipment. We report here our development of a computer-based system that we adapted from the system of Byng et al. (9). This has been

implemented on a standard personal Apple Power Macintosh computer with a relatively low-resolution scanner.

For our pilot trial of a GnRHA-based breast cancer chemoprevention regimen we developed a comparative method where the mammographic densities in mammograms obtained before and after use of the regimen could be compared by simultaneous evaluation (11).

In the current project both the expert outlining method (6) and our computer-based threshold method were applied to the mammograms from the GnRHA trial (11). The primary purpose of this was to evaluate whether a more standard method of measuring mammographic densities (the expert outlining method) would also detect the changes noted with our non-standard simultaneous evaluation method. A second purpose was to evaluate whether the standard expert outlining method could be replaced by a non-expert computer-based threshold method.

SUBJECTS AND METHODS

Mammograms

Women in this study were participants in a clinical trial of a GnRHA-based regimen designed to reduce breast cancer risk (11). Twenty-one women aged 25 to 40 were randomly assigned in a 2:1 ratio to the contraceptive regimen (14 women) or to a control group (7 women). One woman was later removed from the contraceptive group because of poor compliance with the regimen. A second woman in the contraceptive group had breast implants, and her mammograms were for technical reasons found not suitable for inclusion in this study. Women in the contraceptive group received: (a) 7.5 mg leuprolide acetate depot (Lupron Depot®) by intramuscular injection every 28 days, (b) 0.625 mg oral conjugated estrogen (Premarin®) 6 days out of 7 every week, and (c) 10 mg oral medroxyprogesterone acetate (Provera®) for the last 13 days of every fourth 28-day cycle. The regimen was designed to minimize exposure of the breast epithelium to estrogen and progestin while preserving the beneficial effects of estrogen on cardiovascular disease risk and still preventing endometrial hyperplasia.

Mammograms were obtained at baseline and after 12 months on study. The craniocaudal mammograms were used to study mammographic changes from baseline to 12 months in the treated and control groups. Four mammograms per woman were used (right and left breasts at

baseline and 12 months). All 'readings' of mammograms (by all 3 methods described below) were done masked as to baseline or 12-months status and as to being from treated or control women.

Simultaneous Evaluation Method

This is the method we originally used (11). Each baseline film was matched with the corresponding 12 months film. The films in each pair were designated 'A' and 'B' at random. Each pair was then presented to two experienced radiologists (YRP, JGP) familiar with reading mammograms. Each pair of films were independently compared by each reader in terms of: (1) clarity of fibrous septae (trabeculae), where greater clarity is associated with less density; (2) amount of nodular collections of glandular tissue; and (3) amount of confluent areas, where breast tissue is arranged as diffuse sheets. The results were recorded on a 5-point scale (-2, -1, 0, 1 and 2) as a difference between the 12-months and baseline mammograms, where a negative value indicated a reduction in density from baseline to 12 months. The individual subject's average change scores (both views) for each reader were calculated for all 3 questions combined. For the comparison between this method and the other 2 methods, the results from only one of the radiologists were used. (The results were essentially unaffected by the particular choice of radiologist.)

Expert Outlining Method

One of us (MS), very experienced with the method of outlining densities (6-8), outlined the area of the breast that contained mammographic densities on each of the 76 mammograms. Each mammogram (with the outline) was subsequently digitized and the outlined area of the breast containing densities measured (effectively in terms of computer screen pixels). Finally, the total area of the breast was outlined on the digitized image by one of us and measured (see below for details). After estimating the % of the breast with densities separately for the left and the right breast, the average % densities for the two breasts was calculated.

For the expert outlining method 2 measures of effect were calculated: (1) the difference between the 12-months and baseline measures of the % of the breast containing densities (%

densities), expressed as a % of baseline measure; and (2) the difference between the 12-months and baseline density areas (absolute densities), again expressed as a % of the baseline measure.

Computer-Based Threshold Method

All mammograms were digitized using an Omnimedia XRS 6cx scanner (Lumisys, Sunnyvale, CA). This scanner creates an 8 bit (256 shades) gray scale image that is linear in the optical density range of 0-2.8. The mammograms were scanned at a resolution of 150 pixels per inch (59 dots per cm). A pixel value of 0 represents the darkest (black) shade in the image, a value of 255 the lightest (white) value.

The digitized images were analyzed using a MacOS compatible personal computer system consisting of an Apple Power Macintosh 7100/66 and an Apple Multiple Scan 17 video monitor. The video display was set for 24 bit color mode at a resolution of 832 × 624 pixels. In 24 bit color mode, each screen pixel is represented and stored as three 8 bit fields, one each for red, green, and blue (RGB). Gray is achieved by setting the three RGB fields to the same value. The 8 bit gray-scale images were converted to equivalent 24 bit images for display purposes by setting each of the RGB fields of a 24 bit pixel equal to the value of the corresponding 8 bit pixel.

The software used for measuring densities was developed by one of us (MA). The program provides numerous tools for image processing and interactively defining regions of interest. One tool instantaneously applies a yellow tint to any interactively selected subrange of a gray scale image. The yellow tint is applied non-destructively by setting the red field of each RGB pixel to 0 while leaving intact the green and blue fields. The original gray is restored by simply resetting the red field equal to either the green or blue (since all three were originally equal).

On the digitized mammographic image displayed on the screen, the reader first defines a region of interest (ROI) for analysis; this region includes all of the breast shown on the mammogram, but specifically excludes the pectoralis muscle (same area used for Wolfe's method). The reader then uses the tinting tool to apply a yellow tint to gray levels above some threshold X (i.e. pixels corresponding to 8 bit gray levels $\geq X$ and ≤ 255). The reader searches for the best threshold where all pixels $\geq X$ are considered to represent mammographic densities. The

software counts within the defined ROI both total number of pixels and the number of tinted pixels. The fraction (%) of the breast with densities is taken as the ratio of the tinted area to total area of ROI. After estimating the % of the breast with densities separately for the left and the right breast, the average % densities for the two breasts is calculated.

This method was implemented by one of us (GU), a medically trained epidemiologist who first underwent a training session with MS 6 months prior to conducting the actual readings. In this session MS described the expert outlining method of identifying densities and illustrated the method using 14 of the 76 mammograms. GU outlined an additional 3 mammograms, under the critique of MS. She carried out all the computer-based readings for the study 6 months after completing the training session. The mammograms were read 3 times. For the comparison between the methods described here, the results from the first of these 3 readings were used.

Statistical Methods

In the tables we show the difference in density score at 12 months minus the density score at baseline, using both differences in absolute areas and differences in % of breast with densities.

The statistical significance of the difference between the 12-months and baseline mammograms within each group (GnRHA-regimen and control) was evaluated using Wilcoxon's non-parametric signed ranks test (12) . The statistical significance of the differences in the mammographic changes between the contraceptive group and the control group was evaluated using Wilcoxon's non-parametric rank sum test (12).

Pearson's correlation coefficients were calculated between the expert outlining method and the computer-based threshold method, and between the multiple readings performed by the same person using the computer-based threshold method.

Statistical significance levels (p-values) quoted are two-sided and written '2p='.

RESULTS

Simultaneous Evaluation Method

With the simultaneous evaluation method, there was a reduction in densities in 10 of the 12 women in the treatment group after 12 months, compared to a reduction in 3 of the 7 control women (Table 1). The largest reductions were almost exclusively concentrated in the GnRHA-

based regimen group. With this restricted data set and using only 1 reader, the difference between baseline and 12-months mammograms was statistically significant in the treatment group (2p=0.010). The change in the treatment group was also statistically significant when compared with the change in the control group (2p=0.042).

Expert Outlining Method

With the expert outlining method, there was a reduction in % densities in 11 of the 12 women in the treatment group after 12 months, compared to a reduction in 3 of the 7 control women, and the largest reductions were again almost exclusively concentrated in the GnRHA-based regimen group. The reduction in densities in the treatment group was highly statistically significant (2p=0.002), but the difference between the treatment group and the control group did not quite reach conventional statistical significance (2p=0.069).

The results when evaluating differences in absolute densities estimated with the expert outlining method were very similar to the results when evaluating differences in % densities (data not shown). There was a statistical significant reduction in densities between baseline and 12-months mammograms in the treatment group (2p=0.012), and the change in the treatment group was statistically significant when compared with the change in the control group (2p=0.025).

Computer-Based Threshold Method

The computer-based threshold method showed a reduction in % densities in 9 of the 12 women in the treatment group after 12 months, compared to a reduction in 4 of the 7 control women, and the largest reductions were again almost exclusively concentrated in the GnRHA-based regimen group. The reduction in % densities in the treatment group was statistically significant (2p=0.027); but the difference between the treatment group and the control group did not quite reach conventional statistical significance (2p=0.083).

The results were, as with the expert outlining method, similar for absolute densities.

Correlations Between the Three Different Methods

The correlations between the simultaneous evaluation method and the expert outlining method were 0.68 (% densities) and 0.76 (absolute densities). The correlations between the results from the simultaneous evaluation method and the computer-based threshold method were 0.62 (% densities) and 0.66 (absolute densities).

The correlations between the difference results from the expert outlining method and the computer-based threshold method were 0.64 for % densities and 0.70 for absolute densities. The correlation coefficients between these two methods for measuring densities (not differences) for all the 76 mammograms are shown in Table 2. There was a high correlation between the two methods, and the 3 readings made with the computer-based threshold method were also highly correlated.

DISCUSSION

Both the expert outlining method of Wolfe et al. (6) and our computer-based threshold method showed high consistency with the previous method we had used to distinguish baseline mammographic densities from mammographic densities after 12 months on the GnRHA-based regimen (11). The 3 methods gave very similar results with no clear difference in statistical power between our original simultaneous evaluation method and Wolfe's (expert) outlining method. There may be a slight loss of statistical power with the computer-based (non-expert) system.

We have not presented data on intra- and inter-observer reliability for the simultaneous evaluation method. Extensive use of this method would warrant collection of such data.

Both the expert outlining method and our computer-based threshold method clearly detected changes in mammographic densities.

The results from the computer-based threshold method correlated highly with those using the expert outlining method. This is in accordance with what Byng et al. found with their computer-based threshold method (9).

For epidemiological purposes, an advantage of the computer-based threshold method is that once the images have been digitized, they can be read at any time, one or multiple times, in whichever order desired. Further, the computer-based threshold method requires less training than both the expert outlining method and the simultaneous evaluation method.

The epidemiologist using the computer-based threshold method was trained on a subset of the 76 mammograms that were later used in the study. The number of mammograms and 6 month time lapse since training made it most unlikely that the previous training film results influenced the subsequent 'real' reading results of these mammograms. Further, the correlation between the expert outlining method and the computer-based threshold method was high, and could not be explained on the basis of a small subset of the mammograms. It is thus most unlikely that the results shown here are materially affected by this.

Although we have not demonstrated that our computer-based threshold method can predict breast cancer risk in a population-based study, the high correlations with the expert outlining method suggests that it will be similarly effective in predicting breast cancer risk.

The expert outlining method has been strongly associated with breast cancer risk in previous studies. Saftlas et al. (7) used the expert outlining method on craniocaudal mammograms to measure the percentage of the breast containing densities. Categorizing %-densities into approximate quintiles they found that successive quintiles had relative risks of 1.7, 2.5, 3.8 and 4.3 ($2p<0.0001$). Similarly, applying the expert outlining method to data from the Breast Cancer Detection Demonstration Project, Byrne et al. found that compared with women who had no mammographic densities, women with 75% or more densities had more than 4-fold elevated risk of breast cancer (95% CI 3.1-6.1) (8).

Boyd et al. (10) used the computer-based threshold method of Byng et al. (9) and found that women with 75% or more densities had a 4-fold elevated risk of breast cancer compared to women with no densities ($2p=0.0001$).

The results presented here for the computer-based system were from a single reader. We had 2 'naïve' readers conduct mammographic densities assessments after only minimal training on 10 mammographic images. Despite this minimal exposure to mammographic density readings, these 2 observers obtained correlation coefficients with the expert outlining method on the 76 mammograms of approximately 0.7. One of the problems these 'naïve' readers had was to compensate for differences in quality and exposure of the mammogram. This was easier for more experienced readers. However, Byng et al. (9) have reported that a naive observer can

perform as well as a highly trained one. We are currently in the process of training other readers on an extensive set of films.

Technical Issues

In this study all mammograms were scanned on a scanner with 8 bit pixel depth ($2^8 = 256$ shades of gray), and with a resolution of 150 dpi. Standard personal computer monitors can only display 256 levels of gray; if images are scanned at a higher pixel depth, the computer program must convert these down to 256 in order to display the mammographic image. With the type of monitor we used, a 17 inch monitor with pixel size of 0.28 mm (which corresponds to a resolution of approximately 90 dpi), scanning the images at a resolution of 150 dpi appears adequate. We did, however, also scan a subset of the images with a higher resolution scanner, a Cobrascan CX-312T scanner (Radiographic Digital Imaging Inc., Compton, CA). This scanner provides a 12 bit pixel depth (4,096 shades of gray) at a resolution of 300 dpi. This did not improve the computer images when displayed on a conventional Apple monitor.

Significance of mammographic density reductions

There is substantial epidemiological and experimental evidence that ovarian hormones (in particular, estrogens, and possibly progesterone) increase the risk of breast cancer (13-15). The GnRHA-based regimen attempts to reduce the levels of estrogen and progestogen to a minimum, while still preserving the essential beneficial effects of estrogen. Blocking of ovarian function is achieved through the use of the GnRHA; sufficient estrogen is given to prevent hypo-estrogenic symptoms (such as hot flashes); and intermittent progestogen is given to prevent any estrogen-induced endometrial hyperplasia. The fact that mammographic densities are reduced after a year on this regimen suggests that such a regimen may protect against breast cancer (11). In that regard it is interesting to compare the density reduction obtained with the GnRHA regimen with that obtained in a two year randomized trial of a low-fat, high-carbohydrate diet; the GnRHA regimen resulted in a more than three times larger reduction (21.6%) than the diet (6.1%) (16).

Conclusion

We have demonstrated that both the standard the expert outlining method, and our new computer-based threshold method detected the mammographic density reductions induced by the GnRHA-based regimen. The computer-based threshold method of determining mammographic densities was found to be highly reproducible and correlated well with the expert outlining method. The possibility of using this computer-based threshold method for evaluating the effects of other preventive regimens should be explored further.

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Table 1. Changes in mammographic densities between baseline and 12-months in women treated with a GnRHA based contraceptive regimen and controls assessed by 3 different methods.

Group	Person number	Simultaneous evaluation method (change score)*	the expert outlining method (% change in % densities)	Computer-based threshold method (% change in % densities)**
Treated	1	-1.67	-38.0	-38.1
	2	-1.33	-27.9	-37.1
	3	-1.33	-38.9	-8.3
	4	-1.00	-70.8	-79.1
	5	-1.00	-39.1	-31.4
	6	-0.92	4.7	-24.1
	7	-0.67	-34.7	-39.5
	8	-0.58	-30.2	-44.4
	9	-0.58	-22.5	17.6
	10	-0.17	-2.3	8.2
	11	0.25	-13.7	-0.9
	12	0.67	-0.1	18.2
	mean (± s.e.)	-0.69 (± 0.20)	-26.12 (± 6.10)	-21.6 (± 8.43)
	p-value for change***	0.010	0.002	0.027
Control	1	-0.92	-44.2	-21.2
	2	-0.50	-21.8	24.7
	3	-0.50	28.1	-1.2
	4	0.08	-17.2	-19.4
	5	0.17	3.8	15.7
	6	0.50	33.0	-18.0
	7	1.00	38.2	30.9
	Mean (± s.e.)	-0.05 (± 0.25)	2.83 (± 31.67)	-1.64 (± 8.36)
	p-value for change***	0.94	0.81	0.94
	p-value for difference between groups****	0.042	0.069	0.083

* only data from one of the radiologists are displayed.

** data from the first of three readings.

*** p-values for within group change were calculated using Wilcoxon's signed rank test.

**** p-values for differences between the treated and control group were calculated using Wilcoxon's signed rank sum test.

Table 2 Correlations between the expert outlining method and the computer-based threshold method on 76 mammograms

	Computer-based threshold method reading number:		
	1	2	3
the expert outlining method	0.86	0.89	0.91
Computer-based threshold method reading number:			
1		0.96	0.92
2			0.95

Received 2/8/00

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 26 Jan 00

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2. Point of contact for this request is Ms. Virginia Miller at DSN 343-7327 or by email at virginia.miller@det.amedd.army.mil.

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PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management